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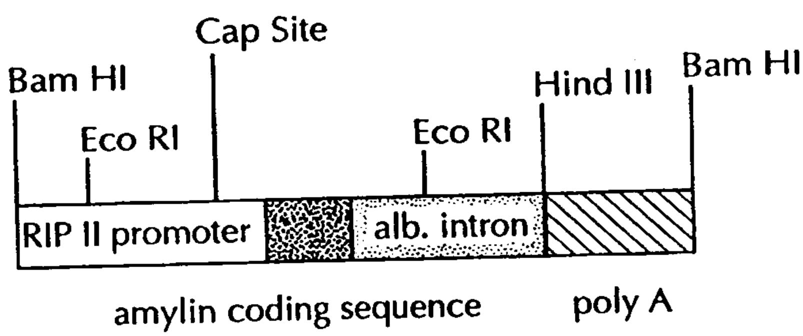

(57) Abstract

The generation of transgenic animal models for testing various treatments of Type II Diabetes Mellitus are described. The DNA construct allows pancreatic β cell - specific expression of human islet associated polypeptide (IAPP) under the regulation of the rat insulin II promoter in both cell lines and transgenic animals. The DNA construct is introduced into animal embryos by microinjection as one or multiple copies or into established cell lines by electroporation. The transgenic animals develop amyloid plaque deposits in the islets of Langerhans in the pancreas, fasting hyperglycemia, glycouria and diabetic glomerulosclerosis at 3 to 5 months of age. The cell lines can be screened for treatments that inhibit expression of human IAPP; the transgenic animals can be screened for treatments that either enhance or inhibit the progression of this disease phenotype.

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TRANSGENIC ANIMAL MODELS FOR TYPE II DIABETES MELLITUS

Background of the Invention

This invention relates to a process for genetic alteration of mammalian cell lines and animals such that they express the protein encoded by the human Islet Amyloid Polypeptide (IAPP) gene. IAPP, formerly known as amylin, is the major protein component of pancreatic islet amyloid that forms in the pancreata of Non-Insulin Dependent Diabetes Mellitus (NIDDM) patients. Recent studies of IAPP structural and functional characteristics suggest that IAPP, along with insulin and other hormones, plays a major role in carbohydrate metabolism. IAPP is produced, stored and secreted by pancreatic β cells in the islets of Langerhans. It can mimic the phenomenon of insulin resistance seen in NIDDM by inhibiting glucose uptake and glycogen synthesis in muscle, and liver tissue. The generation of amyloid deposits in humans is thought to be due to the ability of the center portion of the peptide (amino acids 20-29) to form a β pleated sheet structure. Rodent IAPP differs from human IAPP in that the sequences in this otherwise highly conserved protein between amino acids 20-29 are not conserved and amyloid deposits do not form in rodent pancreata. A working hypothesis is that overexpression of human IAPP leads to insulin resistance in peripheral tissues and in the formation of amyloid deposits.

Transgenic animals, especially mice, have proven to be very useful in dissecting complex systems to generate new information about human disease. Selective expression of human genes in such mice has generated novel model systems to study disease, especially when overexpression of a gene results in a disease state. With such transgenic mice, one can address issues concerning (1) tissue specificity of expression; (2) testing of hypotheses that overexpression of a particular gene leads to disease; (3) the number and identity of tissues/organs that are affected by this overexpression; and (4) effects of various treatments, including drugs, on th progression or amelioration of the disease phenotype.

The generation of transgenic mice that express human IAPP has been reported in the literature, though none of these animals developed a diabetic phenotype. Niles Fox et al. (FEBS Letters 323, 40-44 [1993]) constructed a transgene that fused the rat insulin promoter s qu nce to a genomic DNA fragment containing th entire human IAPP g ne (xons 1-3 and introns 1 and 2). Transgene RNA xpression was detected in pancreas, ant rior pituitary and brain. Although plasma IAPP levels were 5-fold

elevated relative to nontransgenic littermates, no metabolic consequence of this elevation was observed. C.B. Verchere et al. (Diabetologia 37, 725-729 [1994]) used a 600 bp fragment encoding the entire human proIAPP sequence. Their transgenic animals exhibited greater pancreatic content of both IAPP and insulin relative to nontransgenic littermate controls. Increased secretion of both hormones was also detected in perfused pancreas studies. No clinical manifestations of this enhanced storage and secretion were observed. Höppener et. al. (Diabetologia 36, 1258-1265 [1993]) described the generation of multiple transgenic lines that expressed either human or rat IAPP in the mouse endocrine pancreas. Höppener's group used a 703 bp rat insulin II promoter fragment to drive expression of human or rat IAPP from genomic DNA fragments. Plasma IAPP levels were up to 15 fold elevated but no hyperglycemia nor hyperinsulinemia were observed. In a subsequent study, no amyloid plaque was seen to accumulate in vivo but intra- and extracellular amyliod fibrils did form when islets from these transgenics were cultured in vitro under conditions mimicking hyperglycemia (De Koning et al. Proc. Natl. Acad. Sci. 91, 8467-8471 [1994]).

Summary of the Invention

In one embodiment, the present invention is directed to recombinant DNA comprising a non-IAPP promoter, a sequence encoding human IAPP or an active fragment thereof functionally linked to a human albumin intron I encoding sequence, a human GAPDH termination encoding sequence and a human GAPDH polyadenylaton encoding sequence, said DNA resulting in expression of a diabetic phenotype when incorporated into a suitable host.

Especially preferred is recombinant DNA wherein the non-IAPP promoter is selected from the group consisting of promoters for the genes for rat insulin I, rat insulin II, human insulin, mouse IAPP, rat beta cell-specific gluocokinase, glucose transporter 2, human tyrosine amino transferase, human albumin, mouse albumin, rat liver specific glucokinase, and mouse metallothionein.

Also preferred is recombinant DNA wherein said promoter is the rat insulin II promoter.

Especially also preferred is recombinant DNA wherein said sequence encoding human IAPP or an activ fragm nt th reof has th characteristics of cDNA.

Further preferred is recombinant DNA wh r in said s quenc encoding human IAPP or an activ fragm nt thereof has the charact ristics of g nomic DNA.

Also further pref rr d is r combinant DNA wherein said sequence is that of SEQ ID NO: 4.

Also especially further preferred is recombinant DNA wherein said sequence of cDNA is that of SEQ ID NO: 5.

In another embodiment, the DNA sequence encoding human IAPP is replaced by a DNA sequence encoding mouse IAPP or an active fragment thereof, with said mouse DNA preferably having the characteristics of cDNA.

The present invention is also directed to vectors comprising recombinant DNA of the present invention (SEQ. ID NO: 1).

The present invention is also directed to an eukaryotic cell line comprising recombinant DNA of the present invention with preferred cell lines selected from the group consisting of rat insulinoma (RIN) cells, hamster insulinoma (HIT) cells and ß-TC3 mouse insulinoma cells.

The present invention is also directed to transgenic non-human mammals comprising recombinant DNA of the present invention with especially preferred transgenic mammals being mice and rats, said transgenic mammals exhibiting a diabetic phenotype.

In another embodiment, the present invention is directed to a method for treating an animal having disease characterized by an over expression of an IAPP gene product comprising,

administering a therapeutically-effective amount of an inhibitor of the over expression of said IAPP gene product to said mammal.

In yet another embodiment, the present invention is directed to a method of evaluating the effect of a treatment comprising administering said treatment and evaluating the effect of said treatment on the product of over expression of a gene encoding IAPP.

Preferred is the method wherein said treatment is administered to an animal with an especially preferred animal being a human.

The present invention is also directed to a method for determining if a subject is at risk for diabetes or obesity comprising examining said subject for the over expression of an IAPP gene product, said over xpression being indicative of risk.

In still y t anoth r mbodiment, the present inv ntion is direct d to a method of evaluating an animal model for a disorder or diseas stat comprising d termining

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if an IAPP gene in said animal model is expressed at a pr d termined level with a preferred method being wherein said level is higher than the level in a wild type or normal animal.

Brief Description of the Drawings

Figure 1a is a linear map of the RIPHAT transgene. The human IAPP cDNA sequence is depicted in black; the rat insulin II promoter is depicted as a white box, the human albumin intron is a darkly shaded box, the human GAPDH polyadenylation region (labeled poly A) is depicted as a lightly shaded box.

Figure 1b is an enlargement of the ends of the coding region demonstrating the restriction sites that can be used to substitute alternative cDNAs for human IAPP.

Figure 1c is an enlargement of the ends of the promoter region demonstrating the restriction sites that can be used to substitute alternate promoters for RIP II in the RIPHAT transgene.

Figure 2 is a circular map of the plasmid pSV2Dog1. pSVDog1 was constructed by inserting the PCR-modified CAT gene (BspM I/BamH I fragment) downstream of the SV40 promoter (BamH I/Nco I partial fragment from pLuxF3). The resulting plasmid contains the CAT gene coding sequence fused at its 5' end with optimal mammalian translation sequences, and fused at its 3' end with the firefly luciferase 3'-untranslated region and poly A addition site. Expression of the modified CAT gene is driven by the SV40 promoter.

Figure 3 is a circular map of the plasmid pSV2Dog11 containing the human glyceraldehyde 3 phosphate dehydrogenase polyadenylation region used to construct the plasmid pDog 15. pSV2Dog11 was constructed by inserting the PCR amplified human glyceraldehyde-3-phosphate dehydrogenase 3'-untranslated region into Spe I/BamH I digested pSV2Dog1. This places GAPDH 3' non-coding sequences downstream of the CAT coding region.

Figure 3 is a schematic drawing of the cloning strategy used to construct pRIPHAT1, starting with plasmid pDog15.

Figure 4 is an autoradiogram of a genomic DNA southern blot of 6 tail DNAs digested with the restriction endonuclease EcoRI and hybridized to the ^{32}P-labelled human GAPDH fragm nt within th RIPHAT transgen DNA. The 6 lanes repres nt a litt r of animals produced from a cross of RIPHAT transg nic line RG male and FVB/N wild type femal .

Figure 5 is an autoradiogram of a northern blot of total pancreatic RNA isolated from the transgenic lines RHA, RHF and RHG in addition to pancreatic RNA from Human Pancreas and a nontransgenic mouse. The blot was hybridized to a human IAPP cDNA fragment labelled with [alpha ^{32}P]dCTP.

Figure 6 is an electron micrograph of a pancreatic β cell. The arrows outline an intracellular amyloid plaque deposit.

Figure 7 is an electron micrograph showing a 37,000 fold magnification of immunogold staining of intracellular amyloid plaque by means of a rabbit anti human IAPP antibody.

Figure 8 is a graphical representation of the appearance of hyperglycemia in 3 male RHF homozygous mice compared to 3 nontransgenic (FVB/N strain) male mice.

Figure 9 depicts the results of an oral glucose tolerance test performed on 5 week old RHF homozyous transgenic males(litter # RHF11, n= 5) and females (litter # RHF11, n= 3) compared to age-matched nontransgenic FVB/N mice.

Detailed Description of the Invention

The Plasmids:

Plasmid pRIPHAT I(rat insulin promoter human IAPP transgene) (SEQ. ID NO: 1) contains DNA fragments from 5 different sources, three from human genes, the fourth from rat and the fifth being a commercially available plasmid vector. They are the rat insulin II promoter (876 bp); (SEQ ID NO: 2) human IAPP coding sequence (278 bp) (SEQ ID NO: 3), human albumin intron I(720 bp) (SEQ ID NO: 4), and the human glyceraldehyde-3-phosphate dehydrogenase(GAPDH) gene's polyadenylation site and RNA termination region (546 bp) (SEQ ID NO: 5). The commercially available plasmid is Bluescript SK(-) (Stratagene, La Jolla, CA) (SEQ. ID NO: 6). Enzymatic manipulations of recombinant DNA, including ligations, restriction endonuclease digestions, DNA synthesis reactions, and transformations of E. coli were carried out according to well-established procedures as described in Sambrook, J., Fritsch, E.F. and Maniatis, T. **Molecular Cloning: A Laboratory Manual**. 2nd Ed. Cold Spring Harbor Laboratory Press, New York, 1989.

The human albumin intron I (SEQ ID NO: 4) and GAPDH gene fragments (SEQ ID NO: 5) wer obtained from the plasmid pSV2Dog15 by dig stion of this plasmid with Bam HI and ApaLI and isolation of th 1262 bp fragment containing th se two regions. pSV2Dog15 was constructed by David Lloyd and John Thompson of Pfizer, Inc. Th y

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generated the human albumin intron I sequence by polymerase chain reaction (PCR) amplification(Innis, M.A. et al. eds., **PCR Protocols**, Academic Press, New York 1990) of this portion of the albumin gene using human genomic DNA(obtained from Clontech, Palo Alto, CA) and DNA oligomers 18505.022 (sequence 5' CCCTCTAGAAGCTTGTCTGGGCAAGGGAAGAAAA 3') (SEQ ID NO: 8) and 18505.024 (sequence 5' GGGAAGCTTCTAGACTTTCGTCGAGGTGCACGTAAGAA 3') (SEQ ID NO: 9). Since these oligomers included exogenous Xba I sites on their ends, the resulting PCR product was digested with Xba I and inserted into the compatible Spe I site in pSV2Dog11. This plasmid, constructed by David Lloyd, in turn already contained the human GAPDH polyadenylation region. It was also generated by PCR cloning using human genomic DNA as the template and oligomers 18970.246 (sequence 5' CAAACCGGATCCGCCCTGACTTCCTCCACCTGTCAGC 3') (SEQ ID NO: 10) and 18970.244 (sequence 5' CACAACACTAGTGACCCCTGGACCACCAGCCCCAGC 3') (SEQ ID NO: 11) as the PCR primers. The PCR product generated in this manner was digested with Spe I and Bam HI and inserted into SpeI/Bam HI digested pSV2Dog1 (see Figure 2).

The 1262 bp, Apa LI/Bam HI albumin intronI-GAPDH polyA region hybrid fragment was ligated to a 278 bp PCR-amplified DNA fragment containing the coding region for the preproIAPP protein product. This fragment was amplified by using an IAPP cDNA (lambda phage DNA hIAPP-c1, obtained from Sietse Mosselman, Rijksuniversiteit te Utrecht, The Netherlands and described in Mosselman, S. et al., Febs Lett. 247, 154-158 [1989]) as the template and oligomers 19383.288 (sequence 5'GTCATGTGCACCTAAAGGGGCAAGTAATTCA 3') (SEQ ID NO: 12) and 19987.116 (sequence 5' GAAGCCATGGGCATCCTGAAGCTGCAAGTA 3') (SEQ ID NO: 13) as the PCR primers. The resulting 1523 bp fragment was ligated to pSuperLuc (pSL) to generate plasmid pSLA10. pSL is a DNA plasmid containing the luciferase reporter gene (Mosselman, S. et al. FEBS Lett. 271, 33-36 [1990]). In this case, the plasmid was used only for the presence of convenient Nco I and Bam HI restriction sites.

The rat insulin II promoter (SEQ ID NO: 2) and 5' untranslated leader region were generated by PCR amplification of rat genomic DNA (obtained from Clontech, cat # 6750-1) using oligomers 19383.284 (sequenc 5' GTCAGGAATTCGGATCCCCCAACCACTCCAA- GT 3') (SEQ ID NO: 14) and 19383.292 (sequence 5' ACAGGGCCATGGTGGAACAATGA- CCTGGAAGATA 3') (SEQ ID NO: 15).

The oligomer 19383.292 contains a point mutation; and was so designed to introduce an Nco I site at the 3' end of the fragment by altering one nucleotide (A to C) 2 residues 5' of the initiation codon. The 883 bp PCR product was cleaved with Nco I to generate a 175 bp blunt end/Nco I fragment and a 708 bp fragment with 2 Nco I ends. The plasmid pSLA 10 was digested with Xba I. The resulting 5' overhangs were filled in with Klenow polymerase and dNTPs to generate blunt ends. The plasmid was subsequently digested with Nco I and ligated to the 175 bp blunt end/Nco I rat insulin II fragment to generate pSLA11 (see Figure 3).

pSLA11 was digested with Nco I and ligated to the 708 bp Nco I rat insulin II fragment to generate plasmid pSLA12. Proper orientation of the 708 bp Nco I fragment was confirmed by digestion of the plasmid with Eco RI and Bam HI. The chimeric transgene (rat insulin II promoter and 5' untranslated leader, IAPP coding region, albumin intron I, GAPDH polyadenylation region) (SEQ ID NO: 7) was transferred from the pSL backbone to Bam HI -linearized Bluescript SK(-) by partial Bam HI digestion of pSLA12 to generate pRIPHAT I (SEQ ID NO: 1) . The rat insulin II promoter and 5' untranslated leader region and the IAPP coding region were sequenced by the dideoxy chain termination method of Sanger to ensure that no mutations were introduced.

To ensure that the transgene would be expressed in mouse cells, pRIPHAT I (SEQ ID NO: 1) was transiently transfected into βTC cells by means of electroporation as described by Mosselman et. al. (FEBS Lett. <u>271</u>, 33-36. 1990). Total RNA was isolated by established methods (Chomczynski and Sachi, Anal. Biochem. <u>162</u>, 156-159) 24 hours later. The transgene-specific RNA was detected by PCR amplification of the cDNA derived from this total RNA by reverse transcription(Innis, M.A. et al. eds., PCR Protocols, Academic Press, New York 1990). The size and abundance of the PCR product demonstrated that the transgene was expressed and that the human albumin intron portion of the transgene was efficiently spliced out in these cells.

The Stably Transfected Cell Lines

The above described plasmids were stably introduced into RIN and βTC3 cells by electroporation along with a plasmid that confers geneticin(G418) resistance to the recipient cell. βTC3 cells were obtained from Shimon Efrat and Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., and are described in Efrat, S. et al. Proc. Natl. Acad. Sci. 85, 9037-9041 (1988). C lls w r pr pared for electroporation by trypsinization of semi-confluent monolayers, p lleting twice, 1 wash in serum-free RPMI

1640 medium and resuspension in this medium at a concentration of 2×10^7 cells/ ml. Routinely 50 μg of the appropriate plasmid along with 3.3 μg of the selection plasmid pHA2.3neo (confers G418 resistance; Dr. Peter Hobart, Pfizer, Inc.) were added to 0.5 ml of cells in a electroporation cuvette (Bethesda Research Labs [BRL], Gaithersburg, MD) and subjected to 250v/cm Field at 800 μF and low resistance setting on a BRL Cell-Porator electroporation unit. The cells were allowed to rest for 2 minutes. They were then diluted with 2 volumes of RPMI 1640 10% fetal bovine serum and transferred to T25 flasks. After 36 hrs, viable cells were transferred to 6 well cluster dishes and grown at a concentration of 2×10^5 cells in selection medium (same as above with 500 μg/ml active Geneticin). Colonies appeared after 3 weeks and they were isolated by established methods using trypsin and grease-coated porcelain cloning rings. Clones which survived this procedure were grown to mass culture, frozen and stored in liquid nitrogen. Confirmation of transgene expression was obtained by PCR amplification of cDNA derived from the clones' total RNA. In addition, a radioimmunoassay (Peninsula labs kit # RIK-7321, Belmont, CA) was performed on both total cell protein and surrounding medium to confirm both increased IAPP content and secretion.

Transgenic Mice.

Embryos from mouse strain FVB/N (Taketo, M. et al. Proc. Natl. Acad. Sci 88, 2065-2069 [1991]) were injected with linear DNA fragments that were isolated from the plasmid described above. The 2395 bp RIPHAT DNA fragment was released from its plasmid by cleavage with the Xba I and Xho I restriction endonucleases. The 2395 bp transgene fragment was isolated by electroelution (65V, 3 hrs.) after 2 rounds of agarose (0.9% GTG agarose, FMC Bioproducts, Rockland, Me) gel electrophoresis of the reaction digest. The fragment was further purified on a Schleicher and Schuell Elutip-d column following manufacturer's Elutip-d Basic Protocol for DNA purification prior to being injected into the embryos. Injection of the embryos was carried out according to published procedures, as outlined in Hogan, B. et al. **Manipulating the Mouse Embryo** Cold Spring Harbor Laboratories, New York, 1986.

Optimal Expression and Preferred Embodiments

The plasmids as described above can be altered to optimize expression of the transgene such as various insertions, d letions and/or single or multipl bas pair substitutions. This includes singl base pair alterations in the region in front of th initiation codon of lucif rase to optimiz translational efficiency. The promoter r gion

within pRIPHAT 1 can be exchanged for other promoters, such as human IAPP, rat insulin I, mouse insulin, mouse IAPP, rat glucokinase(liver and /or β cell-specific), human gastrin, human or mouse albumin, mouse metallothionein and human tyrosine aminotransferase (Figure 1b). The rat insulin II promoter is depicted as a darkly shaded box, the coding region as a black box, the human albumin intron as a white box and the human GAPDH polyadenylation region as a striped box. IAPP cDNAs of other species such as mouse, or mutated functional forms of human IAPP that retain either the amyloidogenic portions or the portions that induce insulin resistance, can be substituted for the human IAPP cDNA region within pRIPHAT1 (Figure 1c). The transgene DNAs can be injected into other mouse strain embryos and mutants thereof, including db/+, Ob/+, A^{vy} or A^{y} on either a C57BL/6J or C57BL/Ks background. Alternatively, these transgenic mice can be mated to strains with these genetic traits.

The preferred cell lines include βTC3 (Cold Spring Harbor Laboratories), RINm5f (Gazdar, A.F. et al. PNAS 77, 3519-3523 [1980] obtained from W. Chick, U. Mass., Worcester, Massachusetts) and HIT (Santerre, R.F. et al. PNAS 78, 4339-4343 [1981]; obtained from ATCC Rockville MD).

Utility

The stably transfected cell lines can be used to screen drugs for their ability to alter transcription, mRNA levels, translation, accumulation or secretion of human IAPP. In particular, steady state levels of transgene mRNA can be screened in a high throughput fashion using PCR detection methods. The cells can also be used to determine the mechanism of action of candidate drugs that are found to alter the above-mentioned processes.

The transgenic animals can be used to screen drugs for their ability to alter human IAPP levels in cells, tissues, organs and/or plasma. They can also be used to study the pathological consequences of human IAPP overexpression in whole animals.

Experimental

Materials and Methods

Restriction enzymes including ApaLI, Bam HI, Hind III, Nco , Not I, Xba I, Xho I and Eco RI were obtained from New England Biolabs. DNA modifying enzymes including T4 DNA Ligas and T4 DNA Kinase w re obtained from th same source. Bacterial alkaline phosphatase was obtain d from Bo hringer Mannheim. All

commercially obtained enzymes were utilized under conditions described as optimal by the supplier.

Media

The media for growth of E. coli consisted of 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl. The pH was adjusted to 7.5 with 10N NaOH after addition of all the ingredients.

Ethanol Precipitation of DNA

Sodium acetate(NaOAc) from a 3M, pH 5.2 stock solution was added to a DNA sample to bring the final NaOAc concentration to 200 mM. Two and one-half volumes of cold(-20°C) absolute ethanol were added to the one volume of aqueous DNA sample and the sample placed at -70°C for 15 min. or -20°C overnight.

Electrophoresis of DNA

DNA in 10 mM Tris-HCl, pH 7.6 (or Hepes, pH 7.6) , 1 mM EDTA was mixed with 1/5 volume of Loading buffer. Loading Buffer consisted of 30% glycerol, 10 mM Tris-HCl, pH 7.6, 20 mM ethylene diamine tetra acetic acid(EDTA), bromophenol blue. 0.25% (w/v)and xylene cyanol, 0.25%(w/v). DNA was electrophoresed through 0.8-1.2% (w/v) GTG agarose (FMC Bioproducts, Rockland,ME) at 5-10 volts per cm of distance between electrodes in 1X Tris-Borate EDTA (TBE) buffer (89 mM Tris, pH 8.3, 89 mM Borate, 2 mM EDTA).

Electroelution of DNA

DNA bands were removed from gels by cutting out a gel slice containing the band of interest with a clean single-edged razor blade and placing the gel slice in a 0.25 inch diameter dialysis tube (BRL Life Technologies, Inc., Gaithersburg, MD)(length varying with size of gel slice) that was filled with 0.5X TBE buffer and subsequently sealed on both ends with dialysis tube clips. The filled tube was placed in a standard electrophoresis gel box filled with 0.5X TBE buffer and the DNA eluted out of the gel slice onto the inner side of the dialysis tube by applying a voltage of 10 volts/cm of distance between electrodes for 1-3 hours. At the end of this time the buffer solution containing the DNA was transferred to an eppendorf tube, concentrated in a Speed-Vac apparatus (Savant Instruments Inc. Farmingdale, N.Y.) to reduce the volume to a minimum of 100 μl. This volume was appli d to a prepacked G-50 S phadex (Pharmacia, Piscataway, N.J.) spin column and centrifug d for 3 min. at 600 G force.in an IEC tabletop clinical centrifuge (International Equipment Company, Needham HTS,

MA). This allowed removal of th borate salt from the DNA sampl . The sample was then ethanol precipitated and resuspended in 10 mM Tris, pH 7.6 and 1 mM EDTA.

Visualization of DNA Bands in Gels

For DNA to be electrophoresed through agarose gels, 1/10 volume of a 1 mg/ml ethidium bromide (EtBr) solution was added to the sample. DNA bands were visualized after electrophoresis by placing the gel on a UV transilluminator emitting UV light at a wavelength of 320 nm. By this method no destaining procedure was required. DNA bands in gel slices isolated for preparative purposes were electroeluted as described above. The EtBr bound to the DNA was removed by the ethanol precipitation procedure.

Preparation of Bacterial Plasmid DNA

Maxiprep Procedure (for yields of 100 to 2000 μgs of plasmid DNA).

This DNA was prepared by the alkaline lysis procedure described in Maniatis, Molecular Cloning: A Laboratory Manual. 0.5 liters of Luria broth, described under "Media" was inoculated with a 0.1 ml volume of a stationary phase culture of the appropriate E. coli strain.. After adding 125 μg of dry ampicillin powder to the inoculated media, the bacteria were incubated at 37°C with shaking overnight. The next morning the bacteria were pelleted by centrifugation in a Sorvall GS3 rotor (Dupont Instrument Products, Biomedical Division, Newton, CT) at 5,000 rpm for 10 min at 4°C. The pelleted bacteria were resuspended in 20 ml of a solution consisting of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 5 mg/ml lysozyme. The bacteria were left at room temperature for 10 minutes, after which time 40 ml of 0.2 M NaOH/1% SDS was added. The lysed bacteria were allowed to sit at room temperature for an additional 10 minutes after which time we added 20 ml of ice cold 3M sodium acetate, pH 5.2, and the mixture incubated on ice for 10 min. The white precipitate was pelleted by centrifugation in the original tubes for 10 min. at 5,000 rpm in the GS3 rotor. The supernatant was collected and the volume measured. The DNA was precipitated by addition of one equal volume of isopropanol and pelleted by centrifugation in a Sorvall HSA rotor at 7,500 rpm for 15 minutes at 4°C. The pellet was dissolved in 2 ml of 10 mM Tris-HCl and added to a 5 ml polystyrene tube containing 3.10 gm of CsCl. The sample was transf rred to a 3.9 ml Beckman heat seal tub containing 50 μl of a 10 mg/ml solution of EtBr, filled with wat r, placed in a B ckmann TLN 100 rotor and centrifuged for 4 hours at 100,000 rpm.in an Optima TLX Beckman tabletop

Ultracentrifuge (Beckman Instruments, Palo Alto, CA). Bands of plasmid DNA were visible to the naked eye and extracted with a 20G needle and 1cc tuberculin syringe. The EtBr was removed by extracting the plasmid solution with 3 M NaCl-saturated isopropanol. The DNA was subsequently ethanol-precipitated and stored at -20°C.

Miniprep Procedure(for yields of 1 to 20 μgs of plasmid DNA)

This DNA was prepared by the boiling water procedure described in Maniatis, Molecular Cloning: A Laboratory Manual. 1.5 ml of a stationary culture of E. coli was poured into a 1.5 ml eppendorf tube. The remainder of the culture was stored at 4°C. The tube was centrifuged at 12,000 G for 15 seconds in an Eppendorf microfuge at room temperature. The supernatant was removed by aspiration and the pell t resuspended in 0.4 ml of STET buffer: 8% sucrose, 0.5% Triton X100 detergent, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0. 35 μl of 10 mg/ml of lysozyme was added to the resuspended cells. The tube was immediately placed in 100°C water for 1 minute. The tube was removed from the boiling water and centrifuged for 15 minutes at 12,000G. 200 ul of the supernatant was transferred to a new tube and mixed with 200 μl of isopropanol. The sample was stored at -70°C for 15 minutes after which time the precipitated DNA was recovered by centrifugation at 12,000G at 4°C for 10 minutes. The DNA pellet was rinsed with 70% ethanol and allowed to air dry. The pellet was resuspended in 50 μl of 10 mM Tris-HCl. pH 7.6 , 1mM EDTA and stored at 4°C.

Oral Glucose Tolerance Test

Mice to be tested were fasted for > 12 hours; blood samples were obtained from retro-orbital eye bleeds; blood glucose determinations were carried out by use of a "One Touch" Glucometer (Lifescan Inc., Milpitas CA). Blood sampling was carried out before administration of a glucose challenge(t = 0), and 30, 75 and 120 minutes after glucose challenge. The glucose challenge consisted of a 200 mg/ml dextrose solution administered orally at a 1mg/gm body weight dose by means of a 1cc syringe and murine oral dosing needle.

Bacterial plasmid DNA was prepared by the alkaline lysis method described in Maniatis, Molecular Cloning: A Laboratory Manual.

E. coli Transformation.

The bact rial strains consisted of eith r SURE cells obtain d from Stratagene, Inc. or DH5 cells from Beth sda R s arch Labs, Gaith rsburg, MD. Comp tent cells were prepar d according to th $CaCl_2$ method (Maniatis et al. Mol cular Cloning, Cold

Spring Harbor Laboratories, 2nd Ed. 1989), flash frozen in liquid nitrogen and stored at -70°C. Transformation of these strains with plasmids of interest were typically carried out by incubation of 10 ul of ligation mix with 80 ul of competent cells followed by heat shock at 37°C for 2 min and subsequent incubation at 37°C for 1 hr after addition of 0.8 ml Luria broth. Typically, 100 ul of this mixture was plated on LB plates containing 50 ug/ml ampicillin as the selection agent. Colonies were picked after overnight incubation of the plate at 37°C.

Example 1: Construction of pRIPHAT

Construction of pSV2Dog15

A DNA fragment containing the human glyceraldehyde-3-phosphate dehydrogenase polyadenylation/ transcriptional termination region (SEQ ID NO: 5) was generated by polymerase chain reaction(PCR) amplification. The oligonucleotides 18970.244 (SEQ ID NO: 11) and 18970.246 (SEQ ID NO: 10) were incubated with 3 ug of human genomic DNA (Clontech, SanCarlos, CA) under standard PCR conditions: 1 uM primers, 3 ug target DNA, 200 uM dNTPs, 2.5 units Amplitaq DNA polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM $MgCl_2$. The amplification conditions were set at 25 cycles, 1 min at 96°C, 2 min at 58°C, 3 min at 72°C. The resulting 545 bp fragment was digested with 10 units of Spe I and 10 units of Bam HI (37°C/30 min) and ligated to 1 ug of phosphatased, SpeI/ Bam-digested vector DNA pSV2dog1 to generate plasmid pSV2dog11. pSV2Dog11 itself was then digested with Spe I and treated with alkaline phospahatase (0.25 units from Boehringer Mannheim, in 50 mM TrisHCl, pH 8.5 at 55°C for 2 hours). A DNA fragment containing the human albumin intron I (SEQ ID NO: 3) was generated by PCR amplification of 3 ug of human genomic DNA (Clontech) utilizing oligonucleotides 18505.022 and 18505.024 under standard conditions described above. The resulting 740 bp fragment was digested with Spe I and ligated to Spe I -linearized pSV2 Dog11 under standard ligation conditions to generate plasmid pSV2Dog15. The orientation of the intron fragment was confirmed by digestion of pSV2Dog15 with restriction endonuclease Aat I. 20 ug of pSV2Dog15 was digested with 60 units of Bam HI and 22.5 units of Nco I to isolate a Nco I/BamH I fragment by electroelution. Three micrograms of this fragment was subsequently digested with 20 units of Apa LI for 4 hrs. at 37°C. Th resulting digestion products w r separat d on a 0.8% GTG agarose (FMC Bioproducts, Rockland, ME) gel. A 1265 bp Bam HI/Apa LI fragment was r covered by electroelution (standard conditions: 60

V/ 60 minutes in 0.5X TBE(Tris-borate-EDTA pH 8.3) with th gel slice in a dialysis bag). The eluted DNA was purified by spin column chromatography using G50 resin followed by ethanol precipitation. This fragment contained the human albumin intron fused on its 3' end to the human GAPDH polyadenylation region.

Construction of pSLA10

A portion of the human IAPP cDNA (SEQ ID NO: 2) containing only the protein coding region of the IAPP message was generated by PCR. Oligonucleotides 19383.288 (SEQ ID NO: 12) and 19383.292 (SEQ ID NO: 15), at a concentration of 1 uM were incubated with 0.1 ng cloned IAPP cDNA Hsiapp1 (Mosselman et al.) as the template. Standard buffer and cycling conditions, as described above, were utilized. The reaction products were extracted with chloroform to remove residual mineral oil and ethanol precipitated. The precipitated DNA was resuspended in 20 ul of 1X NEB 4 restriction endonuclease buffer and 10 units of restriction endonuclease Apa LI. The digestion products were electrophoresed in a 1% GTG agarose gel, visualized by ethidium bromide staining, and recovered by electroelution with a yield of 1.1 ug. This fragment was ligated to the BamHI/Apa LI, 1265 bp fragment from pSV2Dog15 in a total volume of 20 ul in 1X BRL ligation buffer plus units T4 DNA Ligase. The reaction was incubated at 16 C for 3 days. The residual ligase activity present in this reaction after this incubation was heat-inactivated (65°C/10 min.). This ligation reaction was then diluted to 100 ul in the presence of high salt restriction buffer (100 mM NaCl, 10 mm Tris, pH 7.6, 10 mM $MgCl_2$) and digested with 5 units of Nco I for 2 hrs. at 37°C. The resultant fragment (1533 bp) was isolated by electrophoresis followed by electroelution.

The shuttle vector for cloning this fragment was prepared by digestion of 20 ug of the reporter plasmid pSuperluc (pSL) with 9 units of Nco I and 20 units of Bam HI at 37°C for 3 hrs. in a reaction volume of 100 ul. To this reaction, 5 ul of 1M Tris, pH 8.0 and 22 units of alkaline phosphatase were added. The reaction was now allowed to proceed at 50°C for 2 hrs. to remove phosphate groups on the 5' overhangs and thus prevent recircularization of the vector alone in subsequent ligation steps. The phosphatase reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation. The DNA was resuspended in 10 mM Hepes, pH 7.6, 1 mM EDTA (HE) at a concentration of 0.2 ug/ul.

The 1533 bp IAPP cDNA/albumin intron/GAPDH polyA fragment was cloned into Nco I/ Bam HI-cut pSL by ligation of 1.1 ug of the insert to 0.2 ug of the pSL vector in

a volume of 20 ul in 1X BRL ligation buffer and 400 units of T4 DNA Ligase. The reaction was incubated at 16°C overnight. On the following morning, competent E. coli SURE cells (Stratagene, San Diego, CA) were incubated with 10 ul of ligation mix at 0°C for 20 min followed by a 2 min heat shock at 37°C. 0.8 ml of Luria Broth were added to the mix and incubated at 37°C for 60 min. 100 ul of this mix were spread onto a Luria Broth Agar plate containing 200 ug/ ml ampicillin. Sixteen ampicillin-resistant colonies were picked and cultured in liquid broth; half of these cultures harbored plasmids with the proper insert as determined by digestion of miniprep DNAs (as described in Materials and Methods). One culture (miniprep #3) was grown up to 0.5 liter in Luria Broth for a NDA maxiprep and its plasmid was designated pSLA10.

Construction of pSLA11

The next step involved the insertion of the rat insulin II promoter (RIP) (SEQ ID NO: 2) into pSLA10. Because RIP contains an internal Nco I site, this process was carried out in 2 stages. The RIP DNA fragment itself (876 bp: 700 bp of 5' flank plus 176 bp of 5' untranslated leader including the first intron) was synthesized by PCR under standard conditions (above) utilizing 1 uM each of oligonucleotides 19383.284 (SEQ ID NO: 14) and 19383.292 (SEQ ID NO: 15); 3 ug of rat genomic DNA (Clontech) was used as the template. Oligonucleotide 19383.292 contains a single base alteration (A to C) 2 nucleotides 5' of the initiation codon in order to allow ligation of RIP to the IAPP coding region via a Nco I site. The PCR product was chloroform extracted and ethanol precipitated. The DNA was resuspended in 20 ul of 1X NEB (New England Biolabs) 4 restriction buffer along with 5 units of Nco I and incubated at 37°C for 2 hrs. Two DNA fragments were recovered from this digestion by electrophoresis through 1.0% GTG agarose, visualization by ethidium bromide staining and electroelution of th DNA from the gel slices: a 708 bp DNA with 2 Nco I ends containing the transcriptional start site and 5' leader region and a 168 bp blunt end/Nco I fragment containing the 5' most flanking sequence of the rat insulin II promoter.

The plasmid pSLA10 was first cleaved with Xba I. Ten micrograms of pSLA10 was digested with 20 units of Xba I in a volume of 100 ul of 1X high salt restriction buffer (100 mM NaCl, 10 mM Tris, pH 7.6, 10 mM $MgCl_2$) for 2 hrs. at 37°C. Th reaction was stopped by ph nol/chloroform extraction followed by ethanol precipitation. The 5' single stranded DNA overhangs were filled in by a polymerization step: the DNA was resuspended in 10 ul of 10 mM Hepes, pH 7.6, 1 mM EDTA and added to a final

reaction volume of 100 ul containing 10 mM Tris, pH 7.6, 10 mm $MgCl_2$, 50 mM NaCl, 5 units of Klenow enzyme and 25 uM dNTPs. This reaction was allowed to proceed at room temperature overnight. On the following morning, the fill-in reaction was heated at 65°C for 10 min to inactivate the Klenow enzyme; afterwards 2 ul of 5M NaCl and 5 units of restriction endonuclease Nco I were added and the reaction allowed to proceed at 37°C for 2 hrs. The cleavage reaction was stopped by the addition of 20 ul of gel loading buffer; consisting of 30% glycerol, 10 mM Tris-HCl, pH 7.6, 20 mM ethylene diamine tetra acetic acid (EDTA), bromophenol blue; 0.25% (w/v and xylene cyanol, 0.25% (w/v), the resulting mix was electrophoresed through a 0.8% GTG agarose gel. The linear form of the digested pSLA10 plasmid was recovered by electroelution followed by centrifugation through a G-50 spin column and ethanol precipitation. The precipitated DNA was resuspended in 10 ul of 10 mM Hepes, pH7.6, 1 mM EDTA. Two microliters (1 ug) of this solution was incubated with 0.25 ug of the 174 bp blunt end/Nco I fragment of the rat insulin promoter, 5 units of NEB T4 DNA ligase and 1X BRL (Bethesda Research Laboratories) ligation buffer in a final volume of 20 ul and incubated at 16°C overnight. The next morning 10 ul of the ligation reaction were used to transform competent E. coli SURE cells. Miniprep DNA was prepared (as described in Materials and Methods) from cultures of 16 colonies; 2 displayed Bam HI fragments of the correct size. One of these clones was grown as a DNA maxiprep as described in the Materials and Methods for preparation of more plasmid DNA and given the designation pSLA11.

pSLA11 was incubated with 7.5 units of Nco I in a volume of 200 ul in 1X NEB4 buffer at 37°C for 2 hrs. followed by the addition of 10 ul of 1mM Tris pH 8.0 and 22 units of Boehringer Mannheim alkaline phosphatase and further incubation at 50°C for 2 hrs. This was followed by 3 sequential phenol/chloroform extractions and ethanol precipitation. 0.32 ug of this linearized form of pSLA11 was ligated to 0.5 ug of the 760 bp Nco I rat insulin II promoter fragment described above in a volume of 20 ul in 1X BRL ligation buffer containing 20 units of NEB T4 DNA ligase; the reaction was incubated at 16°C overnight. Ten microliters of this ligation reaction were used to transform competent E. coli SURE cells (as described in Maniatis, Molecular Cloning: A Laboratory Manual). Of 8 coloni s that aros from this transformation ev nt, on miniprep DNA pr paration displayed th corr ctly sized bands as det rmined by comparison to DNA mol cular w ight mark rs supplied by Bethesda Research

Laboratories, Bethesda, MD, when digested with restriction endonuclease Eco RI. This plasmid was partially digested with Bam HI and the transgene insert, 2.4 kb in length, was ligated to Bam HI-digested Bluescript SK(-); generating pRIPHAT (SEQ. ID NO: 1)) in order to facilitate DNA sequence determination. One of 5 independently derived clones displayed no inappropriate mutations and was used to prepare transgene insert for microinjection.

Preparation of RIPHAT DNA for Microinjection

Three hundred micrograms of pRIPHAT (SEQ. ID NO: 1) were digested with 300 units each of restriction endonucleases Xba I and Xho I in a total reaction volume of 600 ul at 37°C for 2 hrs. The reaction was stopped by the addition of EDTA to a concentration of 21 mM, followed by the addition of loading buffer and 2 rounds of electrophoresis through a 0.5 % GTG agarose gel. The gel slice containing the 2.4 kb DNA fragment was removed and the DNA isolated by electroelution (65V, 3 hrs.). The RIPHAT transgene fragment (2.4 kb (SEQ. ID. NO: 7)) was further purified utilizing a Schleicher and Schuell Elutip-d column and following the manufacturer's protocol. The yield was 5.6 ug of purified RIPHAT fragment. pRIPHAT1 was deposited with the American Type Culture Collection on April 27, 1995 and received the designation ATCC 69794. This DNA was delivered to the Pfizer transgenic facility for microinjection.

Microinjection of Mouse Embryos and Generation of Transgenic Mice

The microinjection of mouse embryos and generation of transgenic mice was carried out by published procedures. Detailed procedures describing the preparation of mice, the microinjection procedure, the reimplantation of injected embryos, the maintenance of foster mothers, and the recovery and maintenance of transgenic lines can be found in Gordon,J and Ruddle, F., Methods in Enzymology 101, 411-433 (1983). Embryos were isolated from female F1 progeny of strain FVB/N inbred crosses. The actual injection procedure was carried out as described in Wagner, T. et al. PNAS 78, 6376-6380 (1981) except that injected eggs were transferred immediately to donor females instead of 5 day incubations in culture tubes. Mice resulting from th reimplantation events were tested for presence of the transgene in their genomic DNA by slot/Southern blotting of DNA isolated from tail biopsies. Those testing positive wer crossed to nontransg nic FVB/N mice of the pposite sex. Offspring of these crosses were tested for transmission of th transg ne by obtaining tail biopsi s, isolating genomic DNA from th m and PCR amplifying transgen sequences using primers

22018-134-1 (5'-CGAGTGGGCTATGGGTTTGT-3') (SEQ ID NO: 16) and 22018-134-2 (5'-GTCATGTGCACCTAAAGGGGCAAGTAATTCA-3') (SEQ ID NO: 17) to generate a diagnostic 883 bp PCR DNA product.

Establishment of Transgenic Lines

Those offspring testing positive for presence of the transgene were backcrossed to FVB/N mice for establishment of transgenic lines. Injection of 280 FVB/N embryos resulted in generation of 10 RIPHAT founders. Six of these founders were able to transmit the transgene to their offspring, as determined by PCR amplification of transgene sequences from genomic DNA isolated from offspring tail biopsies.

Identification of Lines Expressing the Transgene

Total RNA was prepared from various tissues of offspring from the 6 lines (including pancreas, liver and kidney). The RNA was isolated by polytron (Brinkmann Instruments, Westbury, N.Y.) homogenization of each of the tissues in 2 ml of TRISOLV™ (Biotecx Laboratories, Houston, TX) denaturant for 60 seconds. Addition of 4 ml of chloroform allowed separation of the homogenate into an upper, aqueous phase and a lower phenol/chloroform phase. The RNA was precipitated by addition of an equal volume of isopropanol to the aqueous phase of each homogenate. The isopropanol precipitates were centrifuged at 12,000 G for 10 min at 4°C, washed once with 75% ethanol and allowed to air dry. The RNA samples were resuspended in 200 μl of 1 mM EDTA and their concentration determined by UV spectrophotometry. Northern analysis was carried out as described in Maniatis, utilizing 1.0% GTG formaldehyde agarose gels, blotting to Nylon membranes and hybridizing the blot to a 32^P-labelled DNA fragment that corresponds to the human GAPDH polyadenylation region within the RIPHAT transgene. RIPHAT-specific RNA was detected in lines RHA, RHF and RHC, with lines RHA and RHF displaying 10 fold higher pancreatic expression than line RHC. Line RHF was selected for colony expansion.

Generation of Line RHF Homozygotes

The transgenic hemizygous RHF offspring were subjected to brother-sist r matings to generate nontransgenic to hemizygote to homozygote offspring in a 1:2:1 ratio. Transgenic offspring were identified by PCR analysis of tail biopsies; homozygotes within this group were id ntified by t st-crossing th transgenics to wild-type FVB/N mates and identifying those animals that would generat no nontransgenic

offspring (>20 offspring per putativ homozygote). Homozygotes that were identified in this manner were intercrossed to generate a colony of RHF homozygotes.

Determination of Plasma IAPP and Insulin Levels

Representative nontransgenic littermates, hemizygous and homozygous animals were sacrificed by CO_2 asphyxiation. Whole blood was obtained from Vena Cava puncture of asphyxiated animals with a 20G needle and 1 ml tuberculin syringe. The whole blood was transferred to 1.0 ml Microtainer Plasma separator tubes (Becton Dickinson, Rutherford, NJ) to prevent coagulation and centrifuged at 2000G for 2 min to allow plasma isolation. The plasmas were quick frozen in dry ice and stored at -70°C until assayed.

Use of Line RHF Homozygotes for Drug Screening

Typically, animals are divided into groups of 10 for each dose of a given test compound. Their plasma glucose levels are determined by retro-orbital eye bleeds on day 1 before dosing. Dosing is carried out daily, e.g., at 0.1, 1.0 and 10 mg/kg, for days 1 through 4. On day 5 the animals are bled to determine their fasting plasma glucose levels with the aim of detecting a glucose lowering effect. Alternatively, the animals are subjected to an oral glucose tolerance test (OGTT) to demonstrate improved glucose tolerance. The animals are then exsanguinated in order to measure plasma insulin levels and demonstrate a drop in insulin concentration.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Soeller, Walter C.
Carty, Maynard D.
Kreutter, David K.
(APPLICANTS FOR UNITED STATES OF AMERICA ONLY)
Pfizer Inc.
(APPLICANT FOR ALL OTHER COUNTRIES)

(ii) TITLE OF INVENTION: TRANSGENIC ANIMAL MODELS FOR TYPE II DIABETES MELLITUS

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10017-5755

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US N/A
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5356 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGTTCTTCGG GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG TTCGATGTAA 5100

CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT TTCTGGGTGA 5160
GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG GAAATGTTGA 5220
ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA TTGTCTCATG 5280
AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT 5340
CCCCGAAAAG TGCCAC 5356

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 876 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGATCCCCCA ACCACTCCAA GTGGAGGCTG AGAAAGGTTT TGTAGCTGGG TAGAGTATGT 60
ACTAAGAGAT GGAGACAGCT GGCTCTGAGC TCTGAAGCAA GCACCTCTTA TGGAGAGTTG 120
CTGACCTTCA GGTGCAAATC TAAGATACTA CAGGAGAATA CACCATGGGG CTTCAGCCCA 180
GTTGACTCCC GAGTGGGCTA TGGGTTTGTG GAAGGAGAGA TAGAAGAGAA GGGACCTTTC 240
TTCTTGAATT CTGCTTTCCT TCTACCTCTG AGGGTGAGCT GGGGTCTCAG CTGAGGTGAG 300
GACACAGCTA TCAGTGGGAA CTGTGAAACA ACAGTTCAAG GGACAAAGTT ACTAGGTCCC 360
CCAACAACTG CAGCCTCCTG GGGAATGATG TGGAAAAATG CTCAGCCAAG GACAAAGAAG 420
GCCTCACCCT CTCTGAGACA ATGTCCCCTG CTGTGAACTG GTTCATCAGG CCACCCAGGA 480
GCCCCTATTA AGACTCTAAT TACCCTAAGG CTAAGTAGAG GTGTTGTTGT CCAATGAGCA 540
CTTTCTGCAG ACCTAGCACC AGGCAAGTGT TTGGAAACTG CAGCTTCAGC CCCTCTGGCC 600
ATCTGCTGAT CCACCCTTAA TGGGACAAAC AGCAAAGTCC AGGGGTCAGG GGGGGGTGCT 660
TTGGACTATA AAGCTAGTGG GGATTCAGTA ACCCCCAGCC CTAAGTGACC AGCTACAGTC 720
GGAAACCATC AGCAAGCAGG TATGTACTCT CCAGGGTGGG CCTGGCTTCC CCAGTCAAGA 780
CTCCAGGGAT TTGAGGGACG CTGTGGGCTC TTCTCTTACA TGTACCTTTT GCTAGCCTCA 840
ACCCTGACTA TCTTCCAGGT CATTGTTCCA CCATGG 876

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 278 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCATGGGCAT CCTGAAGCTG CAAGTATTTC TCATTGTGCT CTCTGTTGCA TTGAACCATC 60
TGAAAGCTAC ACCCATTGAA AGTCATCAGG TGGAAAAGCG GAAATGCAAC ACTGCCACAT 120
GTGCAACGCA GCGCCTGGCA AATTTTTTAG TTCATTCCAG CAACAACTTT GGTGCCATTC 180
TCTCATCTAC CAACGTGGGA TCCAATACAT ATGGCAAGAG GAATGCAGTA GAGGTTTTAA 240
AGAGAGAGCC ACTGAATTAC TT CCCCTTT AGGTGCAC 278

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GTGCACGTAA GAAATCCATT TTTCTATTGT TCAACTTTTA TTCTATTTTC CCAGTAAAAT    60
AAAGTTTTAG TAAACTCTGC ATCTTTAAAG AATTATTTTG GCATTTATTT CTAAAATGGC   120
ATAGCATTTT GTATTTGTGA AGTCTTACAA GGTTATCTTA TTAATAAAAT TCAAACATCC   180
TAGGTAAAAA AAAAAGGTCA GAATTGTTTA GTGACTGTAA TTTTCTTTTG CGCACTAAGG   240
AAAGTGCAAA GTAACTTAGA GTGACTGAAA CTTCACAGAA TAGGGTTGAA GATTGAATTC   300
ATAACTATCC CAAAGACCTA TCCATTGCAC TATGCTTTAT TTAAAAACCA CAAAACCTGT   360
GCTGTTGATC TCATAAATAG AACTTGTATT TATATTTATT TACATTTTAG TCTGTCTTCT   420
TGGTTGCTGT TGATAGACAC TAAAAGAGTA TTAGATATTA TCTAAGTTTG AATATAAGGC   480
TATAAATATT TAATAATTTT TAAAATAGTA TTCTTGGTAA TTGAATTATT CTTCTGTTTA   540
AAGGCAGAAG AAATAATTGA ACATCATCCT GAGTTTTTCT GTAGGAATCA GAGCCCAATA   600
TTTTGAAACA AATGCATAAT CTAAGTCAAA TGGAAAGAAA TATAAAAAGT AACATTATTA   660
CTTCTTGTTT TCTTCAGTAT TTAACAATCC TTTTTTTTCT TCCCTTGCCC AGACAAGCTT   720
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 545 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AAGCTTCTAG TGACCCCTGG ACCACCAGCC CCAGCAAGAG CACAAGAGGA AGAGAGAGAC    60
CCTCACTGCT GGGGAGTCCC TGCCACACTC AGTCCCCCAC CACACTGAAT CTCCCCTCCT   120
CACAGTTGCC ATGTAGACCC CCTGAAGAGG GGAGGGGCCT AGGGAGCCGC ACCTTGTCAT   180
GTACCATCAA TAAAGTACCC TGTGCTCAAC CAGTTACTTG TCCTGTCTTA TTCTAGGGTC   240
TGGGGCAGAG GGGAGGGAAG CTGGGCTTGT GTCAAGGTGA GACATTCTTG CTGGGGAGGG   300
ACCTGGTATG TTCTCCTCAG ACTGAGGGTA GGGCCTCCAA ACAGCCTTGC TTGCTTCGAG   360
AACCATTTGC TTCCCGCTCA GACGTCTTGA GTGCTACAGG AAGCTGGCAC CACTACTTCA   420
GAGAACAAGG CCTTTTCCTC TCCTCGCTCC AGTCCTAGGC TATCTGCTGT TGGCCAAACA   480
TGGAAGAAGC TATTCTGTGG GCAGCTCCAG GGAGGCTGAC AGGTGGAGGA AGTCAGGGCG   540
GATCC                                                               545
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2961 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
CTGACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG CGCAGCGTGA   60
CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC TTTCTTCCCT TCCTTTCTCG  120
CCACGTTCGC CGGCTTTCCC CGTCAAGCTC TAAATCGGGG GCTCCCTTTA GGGTTCCGAT  180
TTAGTGCTTT ACGGCACCTC GACCCCAAAA AACTTGATTA GGGTGATGGT TCACGTAGTG  240
GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT GGAGTCCACG TTCTTTAATA  300
GTGGACTCTT GTTCCAAACT GGAACAACAC TCAACCCTAT CTCGGTCTAT TCTTTTGATT  360
TATAAGGGAT TTTGCCGATT TCGGCCTATT GGTTAAAAAA TGAGCTGATT TAACAAAAAT  420
TTAACGCGAA TTTTAACAAA ATATTAACGC TTACAATTTC CATTCGCCAT TCAGGCTGCG  480
CAACTGTTGG GAAGGGCGAT CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG  540
GGGATGTGCT GCAAGGCGAT TAAGTTGGGT AACGCCAGGG TTTTCCCAGT CACGACGTTG  600
TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCACTA TAGGGCGAAT TGGGTACCGG  660
GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATATC GAATTCCTGC AGCCCGGGGG  720
ATCCACTAGT TCTAGAGCGG CCGCCACCGC GGTGGAGCTC CAGCTTTTGT TCCCTTTAGT  780
GAGGGTTAAT TGCGCGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT  840
ATCCGCTCAC AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG  900
CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG  960
GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC 1020
GTATTGGGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC 1080
GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA 1140
ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG 1200
CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT 1260
CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA 1320
GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC 1380
TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT 1440
AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG 1500
CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG 1560
CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT 1620
TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC 1680
TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG 1740
CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC 1800
AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT 1860
AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA 1920
AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT 1980
GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT 2040
GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG 2100
CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG 2160
CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA 2220
ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG 2280
CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCA CTCCG 2340
GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT 2400
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CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA 2460
TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG 2520
GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC 2580
CGGCGTCAAT ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG 2640
GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA 2700
TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG 2760
GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT 2820
GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC 2880
TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA 2940
CATTTCCCCG AAAAGTGCCA C 2961

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2395 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCCCCAA CCACTCCAAG TGGAGGCTGA GAAAGGTTTT GTAGCTGGGT AGAGTATGTA 60
CTAAGAGATG GAGACAGCTG GCTCTGAGCT CTGAAGCAAG CACCTCTTAT GGAGAGTTGC 120
TGACCTTCAG GTGCAAATCT AAGATACTAC AGGAGAATAC ACCATGGGGC TTCAGCCCAG 180
TTGACTCCCG AGTGGGCTAT GGGTTTGTGG AAGGAGAGAT AGAAGAGAAG GGACCTTTCT 240
TCTTGAATTC TGCTTTCCTT CTACCTCTGA GGGTGAGCTG GGGTCTCAGC TGAGGTGAGG 300
ACACAGCTAT CAGTGGGAAC TGTGAAACAA CAGTTCAAGG GACAAAGTTA CTAGGTCCCC 360
CAACAACTGC AGCCTCCTGG GGAATGATGT GGAAAAATGC TCAGCCAAGG ACAAAGAAGG 420
CCTCACCCTC TCTGAGACAA TGTCCCCTGC TGTGAACTGG TTCATCAGGC CACCCAGGAG 480
CCCCTATTAA GACTCTAATT ACCCTAAGGC TAAGTAGAGG TGTTGTTGTC CAATGAGCAC 540
TTTCTGCAGA CCTAGCACCA GGCAAGTGTT TGGAAACTGC AGCTTCAGCC CCTCTGGCCA 600
TCTGCTGATC CACCCTTAAT GGGACAAACA GCAAAGTCCA GGGGTCAGGG GGGGGTGCTT 660
TGGACTATAA AGCTAGTGGG GATTCAGTAA CCCCCAGCCC TAAGTGACCA GCTACAGTCG 720
GAAACCATCA GCAAGCAGGT ATGTACTCTC CAGGGTGGGC CTGGCTTCCC CAGTCAAGAC 780
TCCAGGGATT TGAGGGACGC TGTGGGCTCT TCTCTTACAT GTACCTTTTG CTAGCCTCAA 840
CCCTGACTAT CTTCCAGGTC ATTGTTCCAC CATGGGCATC CTGAAGCTGC AAGTATTTCT 900
CATTGTGCTC TCTGTTGCAT TGAACCATCT GAAAGCTACA CCCATTGAAA GTCATCAGGT 960
GGAAAAGCGG AAATGCAACA CTGCCACATG TGCAACGCAG CGCCTGGCAA ATTTTTTAGT 1020
TCATTCCAGC AACAACTTTG GTGCCATTCT CTCATCTACC AACGTGGGAT CCAATACATA 1080
TGGCAAGAGG AATGCAGTAG AGGTTTTAAA GAGAGAGCCA CTGAATTACT TGCCCCTTTA 1140
GGTGCACGTA AGAAATCCAT TTTTCTATTG TTCAACTTTT ATTCTATTTT CCCAGTAAAA 1200
TAAAGTTTTA GTAAACTCTG CATCTTTAAA GAATTATTTT GGCATTTATT TCTAAAATGG 1260
CATAGCATTT TGTATTTGTG AAGTCTTACA AGGTTATCTT ATTAATAAAA TTCAAACATC 1320
CTAGGTAAAA AAAAAAGGTC AGAATTGTTT AGTGACTGTA ATTTTCTTTT GCGCACTAAG 1380
GAAAGTGCAA AGTAACTTAG AGTGACTGAA ACTTCACAGA ATAGGGTTGA AGATTGAATT 1440
CATAACTATC CCAAAGACCT ATCCATTGCA CTATGCTTTA TTTAAAAACC ACAAAACCTG 1500

```
TGCTGTTGAT CTCATAAATA GAACTTGTAT TTATATTTAT TTACATTTTA GTCTGTCTTC   1560
TTGGTTGCTG TTGATAGACA CTAAAAGAGT ATTAGATATT ATCTAAGTTT GAATATAAGG   1620
CTATAAATAT TTAATAATTT TTAAAATAGT ATTCTTGGTA ATTGAATTAT TCTTCTGTTT   1680
AAAGGCAGAA GAAATAATTG AACATCATCC TGAGTTTTTC TGTAGGAATC AGAGCCCAAT   1740
ATTTTGAAAC AAATGCATAA TCTAAGTCAA ATGGAAAGAA ATATAAAAAG TAACATTATT   1800
ACTTCTTGTT TTCTTCAGTA TTTAACAATC CTTTTTTTTC TTCCCTTGCC CAGACAAGCT   1860
TCTAGTGACC CCTGGACCAC CAGCCCCAGC AAGAGCACAA GAGGAAGAGA GAGACCCTCA   1920
CTGCTGGGGA GTCCCTGCCA CACTCAGTCC CCCACCACAC TGAATCTCCC CTCCTCACAG   1980
TTGCCATGTA GACCCCCTGA AGAGGGGAGG GGCCTAGGGA GCCGCACCTT GTCATGTACC   2040
ATCAATAAAG TACCCTGTGC TCAACCAGTT ACTTGTCCTG TCTTATTCTA GGGTCTGGGG   2100
CAGAGGGGAG GGAAGCTGGG CTTGTGTCAA GGTGAGACAT TCTTGCTGGG GAGGGACCTG   2160
GTATGTTCTC CTCAGACTGA GGGTAGGGCC TCCAAACAGC CTTGCTTGCT TCGAGAACCA   2220
TTTGCTTCCC GCTCAGACGT CTTGAGTGCT ACAGGAAGCT GGCACCACTA CTTCAGAGAA   2280
CAAGGCCTTT TCCTCTCCTC GCTCCAGTCC TAGGCTATCT GCTGTTGGCC AAACATGGAA   2340
GAAGCTATTC TGTGGGCAGC TCCAGGGAGG CTGACAGGTG GAGGAAGTCA GGGCG        2395
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
CCCTCTAGAA GCTTGTCTGG GCAAGGGAAG AAAA   34
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GGGAAGCTTC TAGACTTTCG TCGAGGTGCA CGTAAGAA   38
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
CAAACCGGAT CCGCCCTGAC TTCCTCCACC TGTCAGC   37
```

(2) INFORMATION FOR SEQ ID NO:11:

SDOCID: <WO 9637612A1 I >

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
CACAACACTA GTGACCCCTG GACCACCAGC CCCAGC 36

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
GTCATGTGCA CCTAAAGGGG CAAGTAATTC A 31

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
GAAGCCATGG GCATCCTGAA GCTGCAAGTA 30

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
GTCAGGAATT CGGATCCCCC AACCACTCCA AGT 33

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ACAGGGCCAT GGTGGAACAA TGACCTGGAA GATA 34

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGAGTGGGCT ATGGGTTTGT 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCATGTGCA CCTAAAGGGG CAAGTAATTC A 31

SDOCID: <WO___ 9637612A1 I >

CLAIMS

1. Recombinant DNA comprising a non-IAPP promoter, a sequence encoding human IAPP or an active fragment thereof functionally linked to a human albumin intron I encoding sequence, a human GAPDH termination encoding sequence and a human GAPDH polyadenylaton encoding sequence, said DNA resulting in expression of a diabetic phenotype when incorporated into a suitable host.

2. Recombinant DNA according to claim 1 wherein the non-IAPP promoter is selected from the group consisting of promoters for the genes for rat insulin I, rat insulin II, human insulin, mouse IAPP, rat B cell-specific gluocokinase, glucose transporter 2 human tyrosine amino transferase, human aluminum, mouse albumin, rat liver specific glucokinase and mouse metallothionein.

3. Recombinant DNA according to claim 2 wherein said promoter is the rat insulin promoter II.

4. Recombinant DNA according to claim 1 wherein said sequence encoding human IAPP or an active fragment thereof has the characteristics of genomic DNA.

5. Recombinant DNA according to claim 5 wherein said sequence encoding human IAPP or of active fragment thereof has the characteristics of cDNA.

6. Recombinant DNA according to claim 1 wherein said sequence is that of SEQ ID NO: 1.

7. Recombinant DNA according to claim 5 wherein said sequence of cDNA is that of SEQ ID NO: 3.

8. Recombinant DNA according to claim 1 wherein the sequence encoding human IAPP is replaced by a sequence encoding mouse IAPP or an active fragment thereof.

9. Recombinant DNA according to claim 8 wherein said DNA is cDNA.

10. A vector comprising recombinant DNA according to claim 1.

11. A vector comprising recombinant DNA according to claim 4.

12. A vector comprising recombinant DNA according to claim 5.

13. An eukaryotic cell line comprising recombinant DNA according to claim 1.

14. An eukaryotic cell line comprising recombinant DNA according to claim 4.

15. An eukaryotic cell line comprising recombinant DNA according to claim 5.

16. A cell line according to claim 13 wherein the cells are selected from the group consisting of rat insulinoma (RIT) cells, rat insulinoma (HIT) cells and ß-TC3 mouse insulinoma cells.

17. A cell line according to claim 14 wherein the cells are selected from the group consisting of rat insulinoma (RIT) cells, rat insulinoma (HIT) cells and ß-TC3 mouse insulinoma cells.

18. A cell line according to claim 13 wherein the cells are selected from the group consisting of rat insulinoma (RIT) cells, rat insulinoma (HIT) cells and ß-TC3 mouse insulinoma cells.

19. A transgenic non-human mammal comprising recombinant DNA according to claim 1.

20. A transgenic non-human mammal comprising recombinant DNA according to claim 4.

21. A transgenic non-human mammal comprising recombinant DNA according to claim 5.

22. A transgenic non-human mammal according to claim 19 wherein said animal is a mouse.

23. A transgenic non-human mammal according to claim 20 wherein said animal is a mouse.

24. A transgenic non-human mammal according to claim 21 wherein said animal is a mouse.

25. A method for treating an animal having disease characterized by an over expression of an IAPP gene product comprising,

administering a therapeutically-effective amount of an inhibitor of the over expression of said IAPP gene product to said mammal.

26. A method of evaluating the effect of a treatment comprising administering said treatment and evaluating the effect of said treatment on the product of over expression of a gene encoding IAPP.

27. The method of claim 26, wherein said treatment is administered to an animal.

28. The method of claim 27 wh rein said animal is a human.

29. A method for determining if a subject is at risk for diabetes or obesity comprising examining said subject for the over expression of an IAPP gene product, said over expression being indicative of risk.

30. A method of evaluating an animal model for a disorder or disease state comprising determining if an IAPP gene in said animal model is expressed at a predetermined level.

31. The method of claim 30 wherein said level is higher than the level in a wild type or normal animal.

FIG. 1a

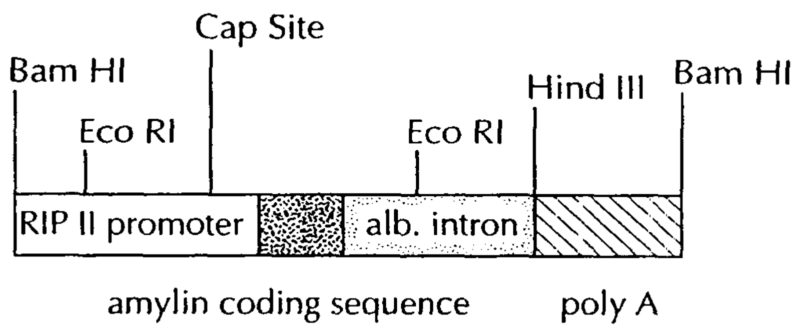

Cap Site
Bam HI
Eco RI
Eco RI
Hind III
Bam HI
RIP II promoter
alb. intron
amylin coding sequence
poly A

FIG. 1B

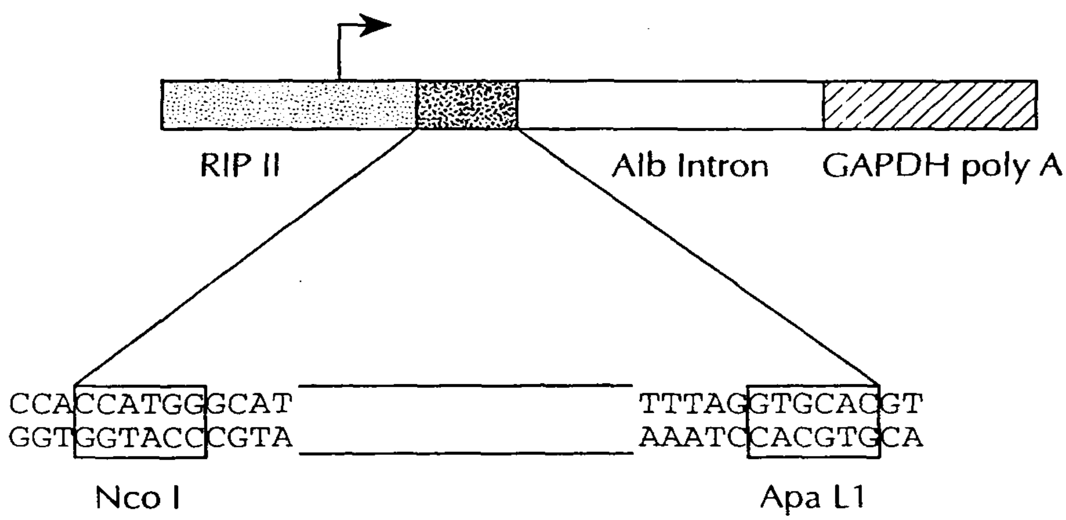
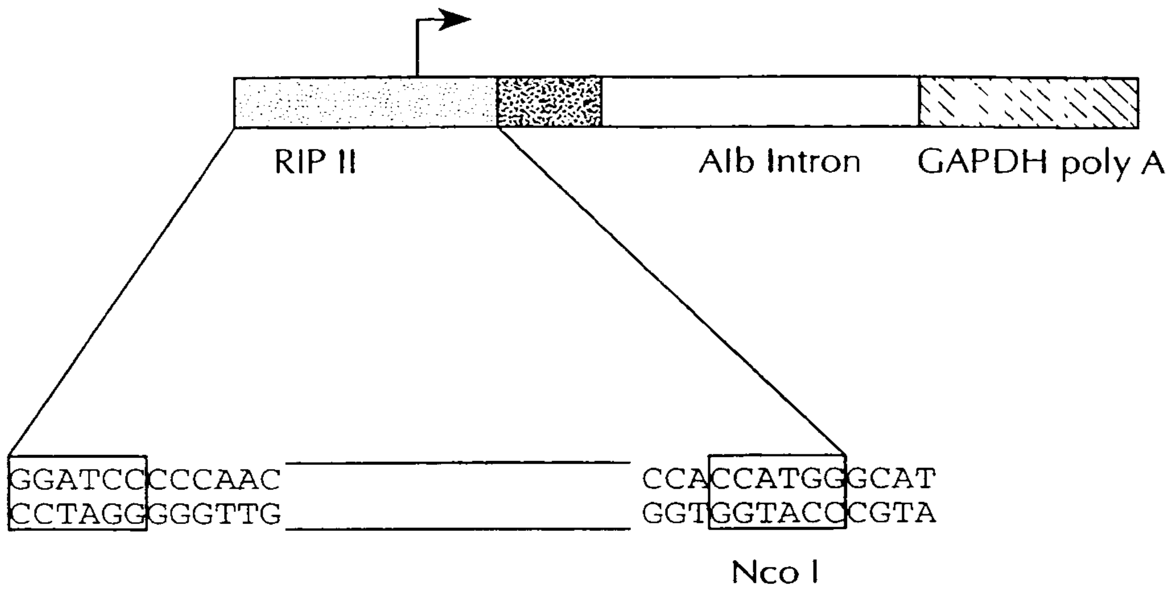

RIP II
Alb Intron
GAPDH poly A
CCACCATGGGCAT
GGTGGTACCCGTA
TTTAGGTGCACGT
AAATCCACGTGCA
Nco I
Apa L1

FIG. 1C


RIP II
Alb Intron
GAPDH poly A
GGATCCCCCAAC
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CCACCATGGGCAT
GGTGGTACCCGTA
Nco I

FIG. 2

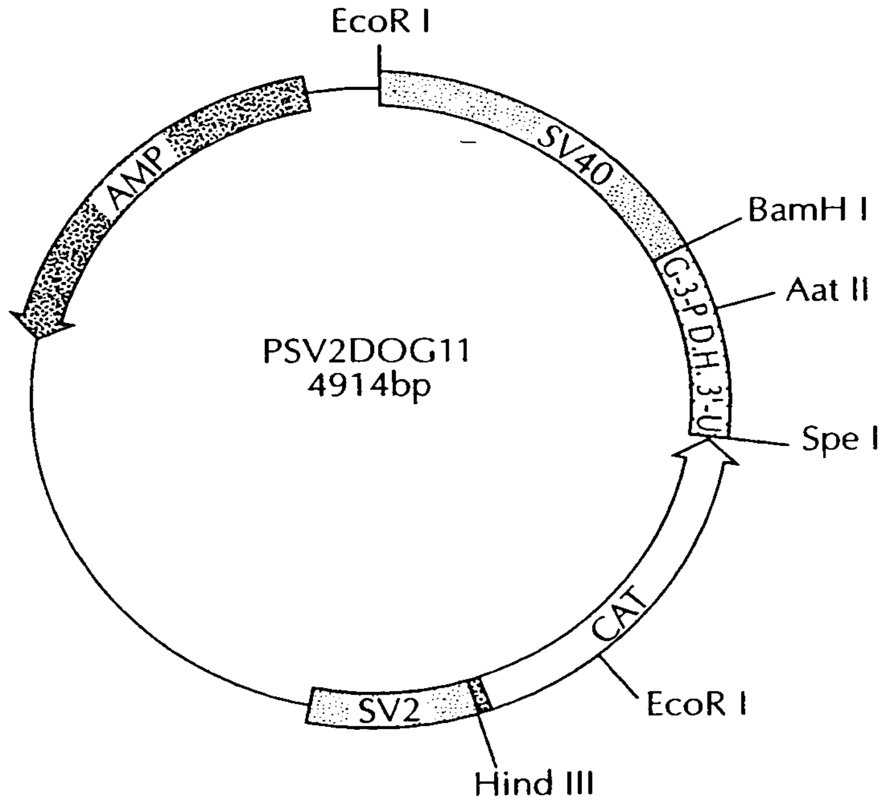

EcoR I
SV40
BamH I
G-3-P D.H. 3'U
Aat II
Spe I
AMP
PSV2DOG11
4914bp
CAT
EcoR I
SV2
Hind III

FIG. 3A

FIG. 3B

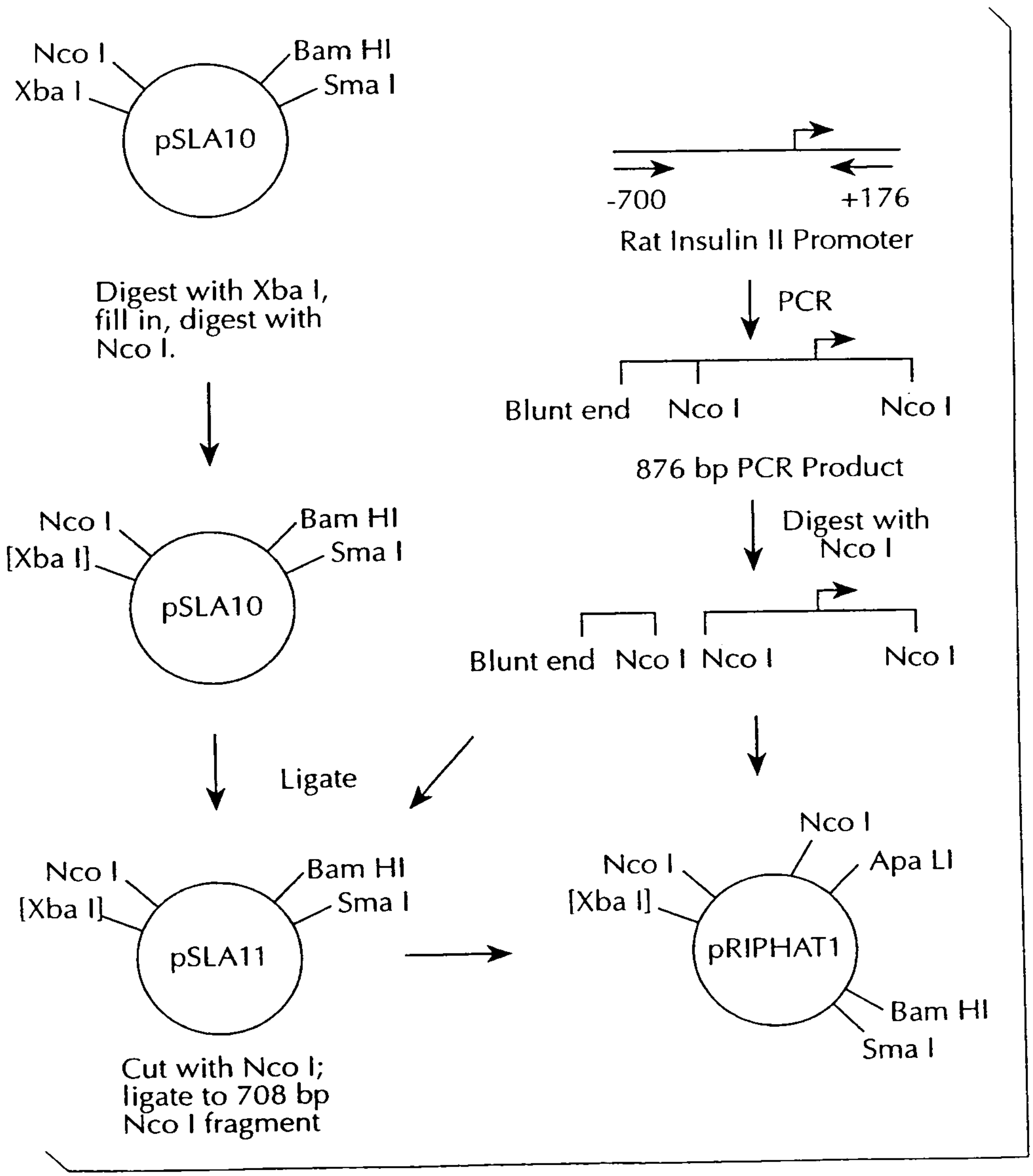

Nco I
Xba I
Bam HI
Sma I
pSLA10
-700
+176
Rat Insulin II Promoter
Digest with Xba I, fill in, digest with Nco I.
PCR
Blunt end
Nco I
Nco I
876 bp PCR Product
Digest with Nco I
Nco I
[Xba I]
Bam HI
Sma I
pSLA10
Blunt end
Nco I
Nco I
Nco I
Ligate
Nco I
[Xba I]
Bam HI
Sma I
pSLA11
Nco I
Apa LI
Nco I
[Xba I]
pRIPHAT1
Bam HI
Sma I
Cut with Nco I; ligate to 708 bp Nco I fragment

FIG. 3A

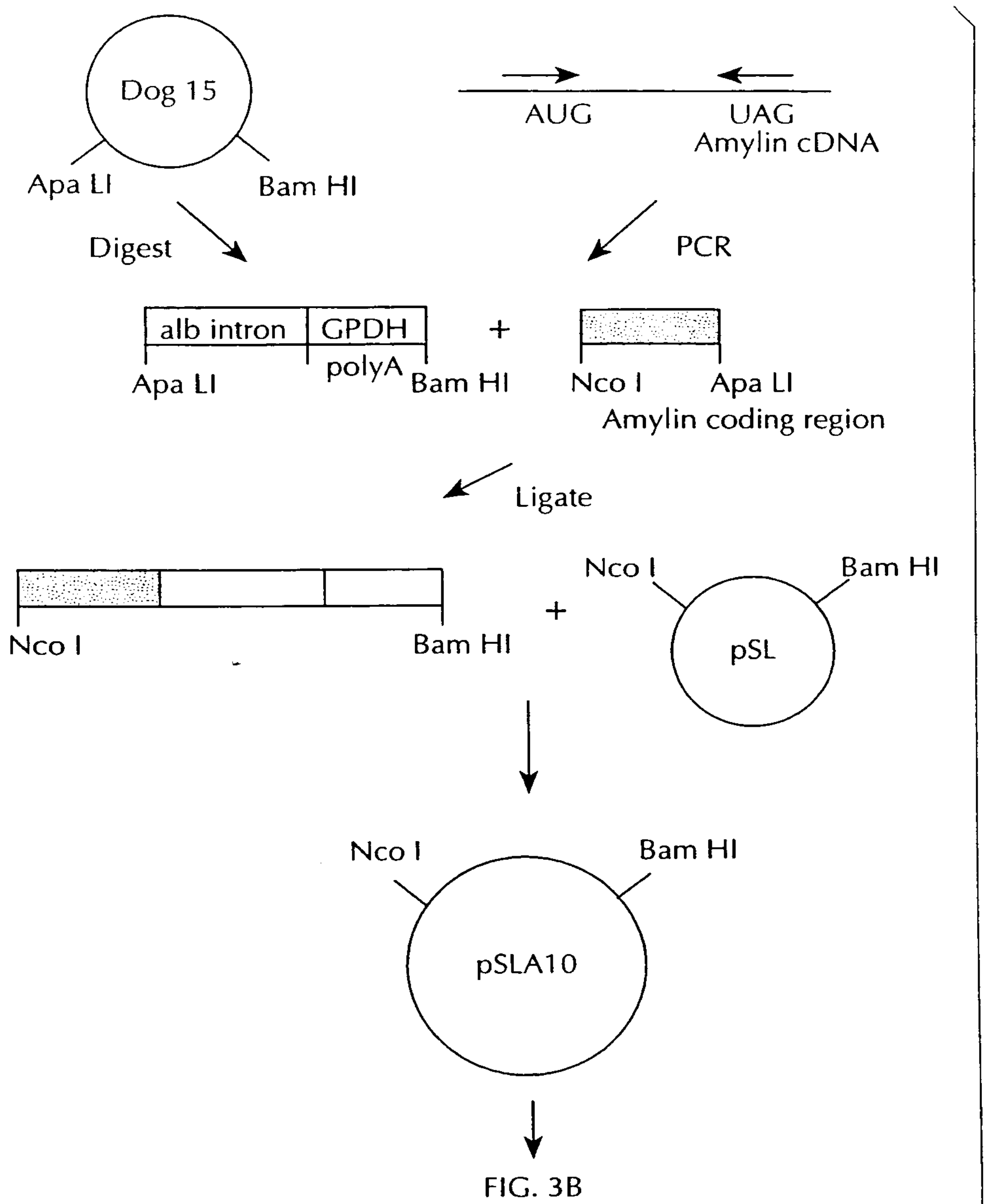

Dog 15
Apa LI
Bam HI
AUG
UAG
Amylin cDNA
Digest
PCR
alb intron
GPDH
polyA
Apa LI
Bam HI
+
Nco I
Apa LI
Amylin coding region
Ligate
Nco I
Bam HI
+
Nco I
Bam HI
pSL
Nco I
Bam HI
pSLA10
FIG. 3B

FIG. 4

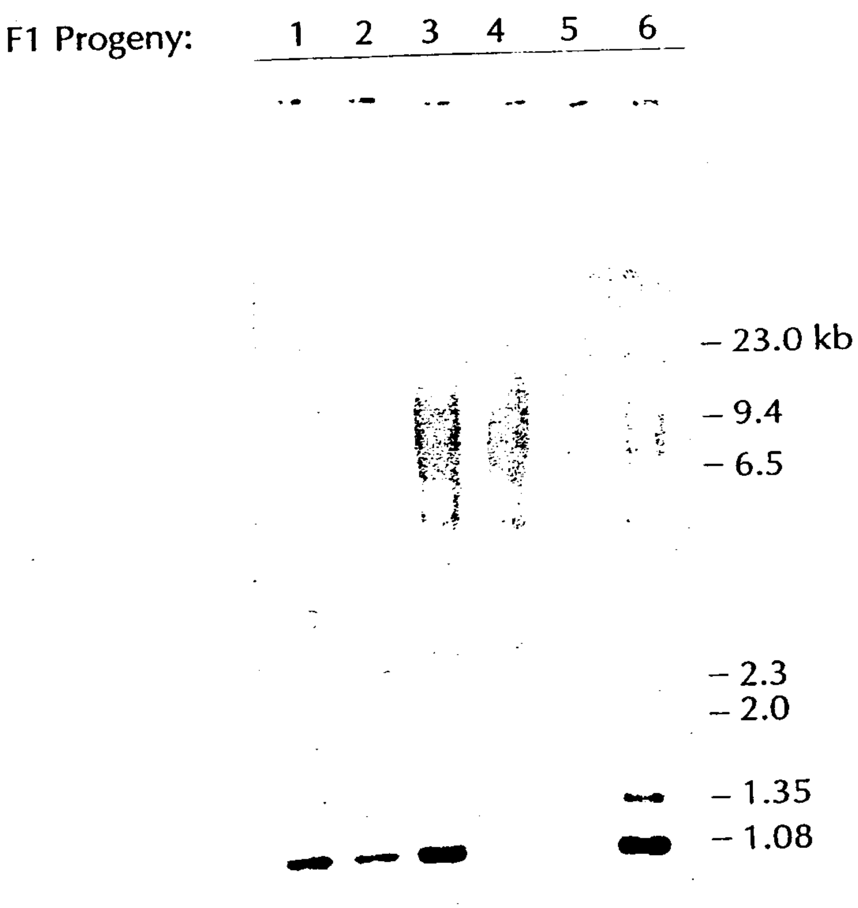


FIG. 5

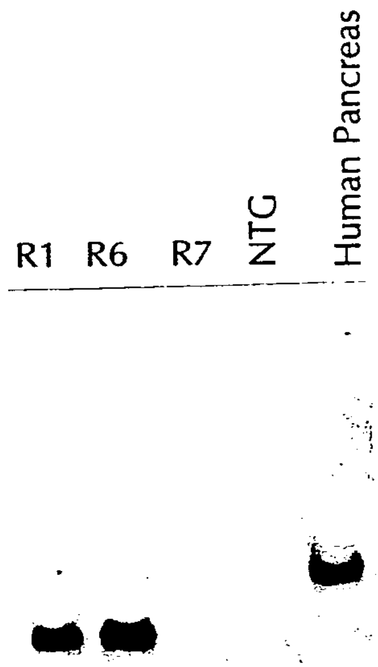


FIG. 6

FIG. 7

FIG. 8

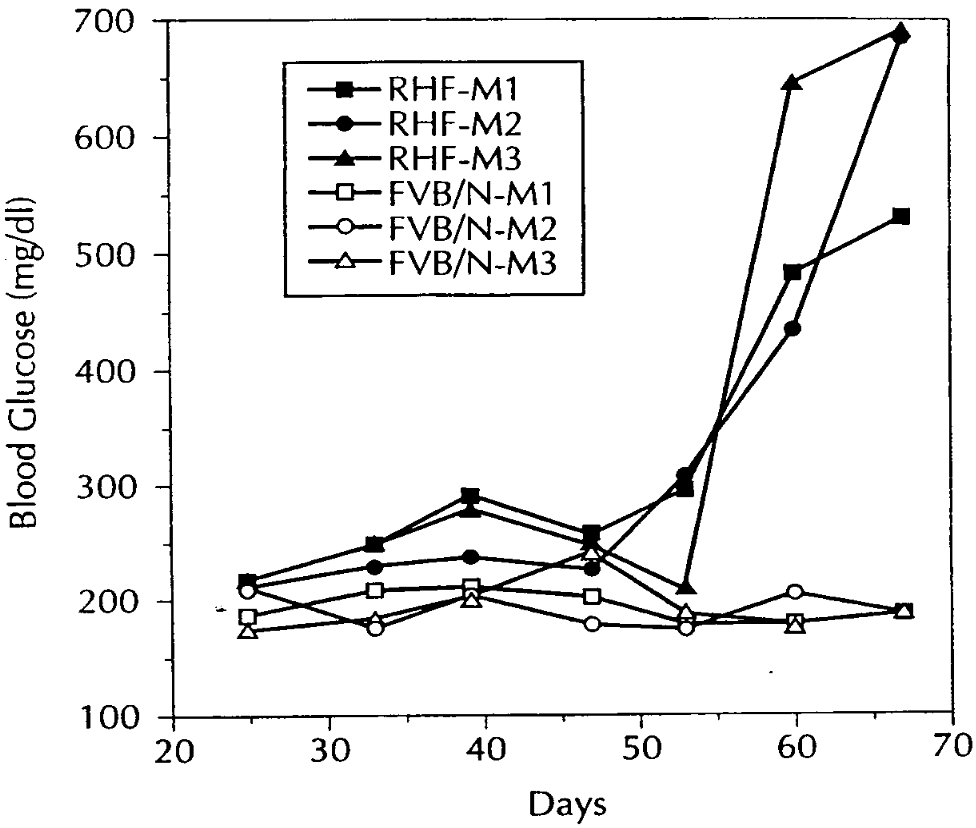

RHF-M1
RHF-M2
RHF-M3
FVB/N-M1
FVB/N-M2
FVB/N-M3
Blood Glucose (mg/dl)
100
200
300
400
500
600
700
20
30
40
50
60
70
Days

FIG. 9A

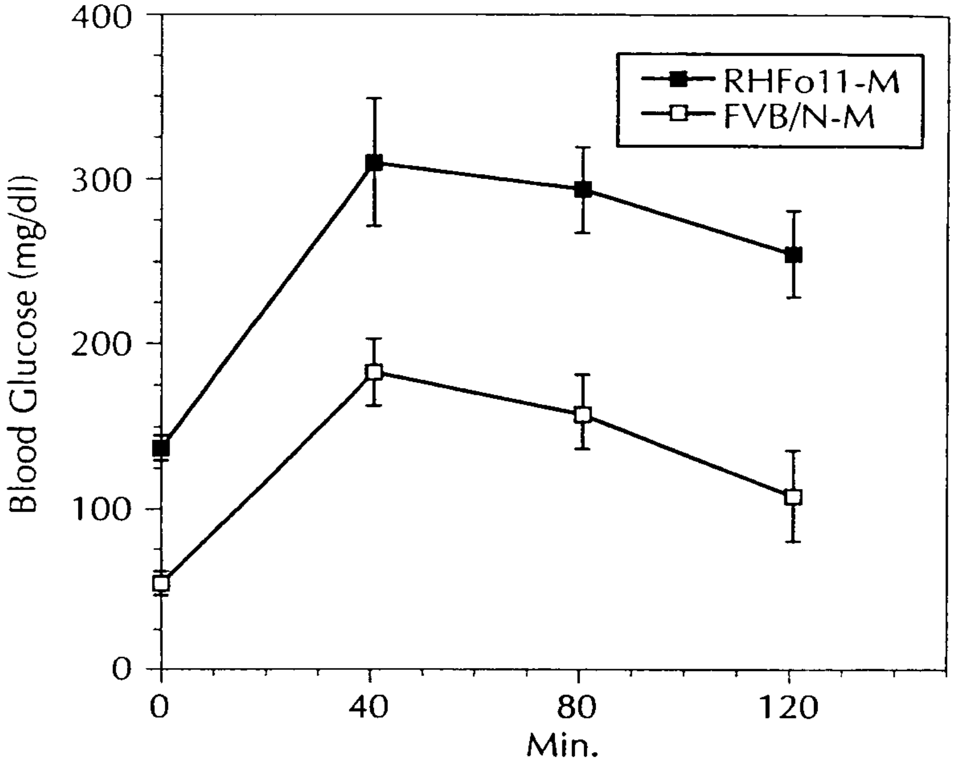
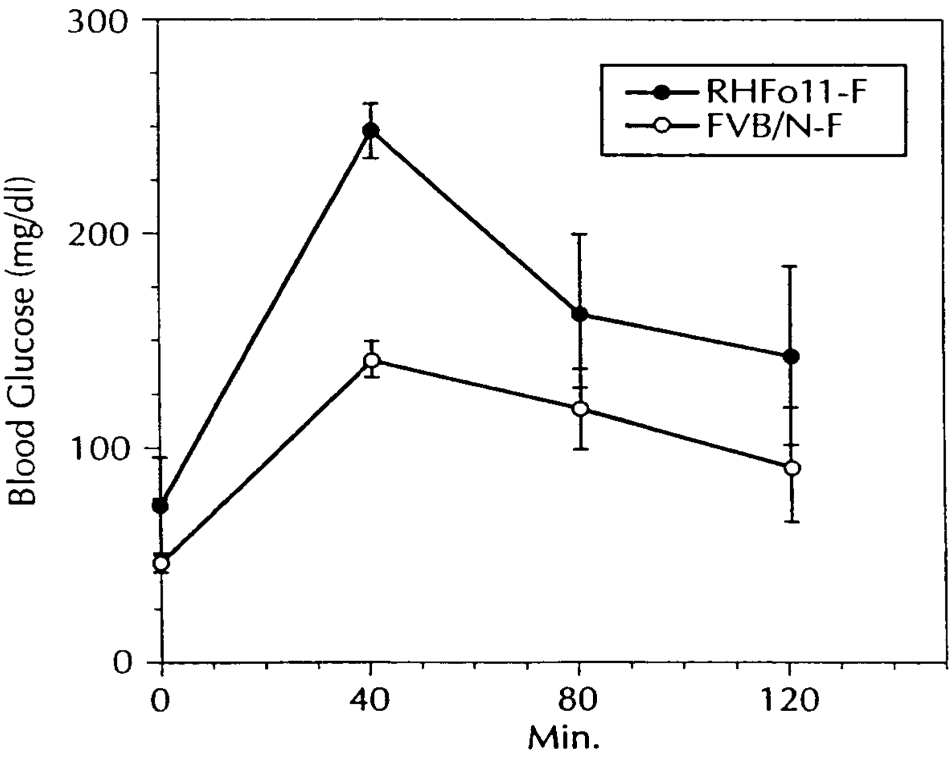

400
300
200
100
0
Blood Glucose (mg/dl)
RHFo11-M
FVB/N-M
0
40
80
120
Min.

FIG. 9B


300
200
100
0
Blood Glucose (mg/dl)
RHFo11-F
FVB/N-F
0
40
80
120
Min.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DIABETALOGICA, vol. 36, 1993, pages 1258-1265, XP000576160 HÖPPENER ET AL: "CHRONIC OVERPRODUCTION OF ISLET AMYLOID POLYPEPTIDE/AMYLIN IN TRANSGENIC MICE:LYSOSOMAL LOCALIZATION OF HUMAN ISLET AMYLOID POLYPEPTIDE AND LACK OF MARKED HYPERGLYCAEMIA OR HYPERINSULINAEMIA" cited in the application	25-31
A	see page 1258,summary	1-24

X	DIABETALOGICA, vol. 37, 1994, pages 725-728, XP000576092 VERCHERE ET AL: "TRANSGENIC MICE OVERPRODUCING ISLET AMYLOID POLYPEPTIDE HAVE INCREASED INSULIN STORAGE AND SECRETION IN VITRO" cited in the application	25-31
A	see page 725,summary	1-24

X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 91, August 1994, pages 8467-8471, XP002009610 DE KONING ET AL: "INTRA-AND EXTRACELLULAR AMYLOID FIBRILS ARE FORMED IN CULTURED PANCREATIC ISLETS OF TRANSGENIC MICE EXPRESSING HUMAN ISLET AMYLOID POLYPEPTIDE" cited in the application	25-31
A	see page 8467,abstract	1-24

X	EP,A,0 408 294 (AMYLIN CORP) 16 January 1991 see page 4, line 39 - page 5, line 22	25-31

X	EP,A,0 503 827 (PFIZER) 16 September 1992 see claims 1-10	25-31

A	JOURNAL OF CELLULAR BIOCHEMISTRY,SUPPLEMENT 0,18 PART A, 1994, XP002009611 SOELLER ET AL: "HUMAN AMYLIN PROMOTER ANALYSIS REVEALS REQUIREMENT FOR BOTH EXON AND INTRON SEQUENCES DOWNSTREAM FROM THE TRANSCRIPTION INITIATION SITE" see abstract C119	1-24

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INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/IB 96/00371

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C12N5/10 A01K67/027 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS LETTTERS, vol. 323, no. 1 2 , May 1993, pages 40-44, XP002009609 FOX ET AL: "HUMAN ISLET AMYLOID POLYPEPTIDE TRANSGENIC MICE AS A MODEL OF NON-INSULIN-DEPENDENT DIABETES MELLITUS (NIDDM)" cited in the application	25-31
A	see the whole document ---	1-24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1996	09.08.95
Name and mailing address of the ISA	**Authorized officer**
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INSDOCID: <WO 9637612A1 I >

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 96/00371

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 87, July 1990, pages 5273-5277, XP002009612 NASRIN ET AL: "AN INSULIN RESPONSE ELEMENT IN THE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GENE BINDS A NUCLEAR PROTEIN INDUCED BY INSULIN IN CULTURED CELLS AND BY NUTRITIONAL MANIPULATIONS IN VIVO" see page 5273,abstract	1-24

P,X	AMERICAN JOURNAL OF PATHOLOGY, vol. 147, no. 3, September 1995, pages 609-616, XP000590832 O'BRIEN ET AL: "HUMAN ISLET AMYLOID POLYPEPTIDE EXPRESSION IN COS-1 CELLS.A MODEL OF INTRACELLULAR AMYLOIDOGENESIS"	25-31
P,A	see page 609,abstract see page 610, paragraph 2	1-24

. 1

BNSDOCID: <WO _ 9637612A1 I >

INTERNATIONAL SEARCH REPORT	International application No. PCT/IB 96/00371

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 25, 27-31 and 26 partially insofar as it concerns in vivo method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the compound/ composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/IB 96/00371

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0408294	16-01-91	AT-T-	128032	15-10-95
		AU-B-	620727	20-02-92
		AU-B-	5953790	06-02-91
		CA-A-	2020786	11-01-91
		DE-D-	69022501	26-10-95
		DE-T-	69022501	01-02-96
		ES-T-	2080802	16-02-96
		JP-T-	4500688	06-02-92
		US-A-	5364841	15-11-94
		WO-A-	9100737	24-01-91
		US-A-	5280014	18-01-94
EP-A-0503827	16-09-92	CA-A-	2063005	16-09-92
		JP-A-	5078255	30-03-93